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Differentiation of animal pox viruses in the incubated hen's egg.

by A. Herrlich and A. Mayr.

The origin and initial occurrence of animal pox are unknown. Its connection with variola and the relationships among the various pathogens have been clarified only in part. It is doubtful that the epizootics described in ancient times and called "smallpox" by the translators actually involved animal pox. The epidemiology, morbid anatomical substrates, immunogenic effect and numerous successful, reciprocal transmissions of pox between animals led to the postulation of a common ancestor of all pox, a "primordial pox." It is no longer possible to decide which type should be considered the "primordial pox." The solution of this frequently discussed and hotly disputed problem is not the subject of this paper. Whether one considers animal pox viruses as having descended from one virus or not, it cannot be denied that these viruses represent biologically well-characterized, independent and stable microorganisms which may be related, but definitely are not identical.

Despite lengthy research in this field, a positive qualitative differentiation of individual animal pox viruses is not possible. This prompted us to develop a method for the differentiation of animal pox viruses based on the incubated chicken egg. Since it has proved itself in our institute during the last few years, and since it may be a valuable aid in special laboratories, the experimental data on which it rests are described in the present paper.

Literature

Cultivation of animal pox viruses in the incubated hen's egg began in 1929, when Gay and Thompson succeeded in propagating vaccinia virus in the yolk sac. In 1951, Woodruff and Goodpasture grew fowlpox virus on the chorioallantoic membrane (CAM). All subsequent cultivation was based essentially on these observations. Inoculation of the CAM became the method of choice. In 1932 Goodpasture, Woodruff and Buddingh described infection of the chorioallantois with vaccinia virus. In 1935, Bierbaum and Gaede succeeded in culturing the virus of pigeonpox through several egg passages. Gins and Kunert reported in 1937 that Kunert had

continued sheeppox viruses through 31 egg membrane passages since 1935.
They were unable to transfer the virus back to the sheep and concluded
that the virus had lost its pathogenicity for sheep during egg passage.
Paschen was the first to inoculate mousepox virus on the CAM in 1936.
Burnet and Lush confirmed these results in the same year. They found
that the infection with ectromelia virus is considerable more effective
at low temperatures of 36-37°C than at the usual physiological tempera-
ture of eggs. Also in 1936, Burnet and Lush applied canarypox virus
(Kikuth-Gollub) to the chorioallantois.

In the wake of these successful cultures in the incubated egg, the
animal pox viruses soon served many investigators as models for the
clarification of numerous virological problems. Of the publications that
furnished fundamental research principles through animal pox infections
of the chick embryo, the most noteworthy include Herzberg's studies of
the propagation of vaccinia virus in the epithelial cells of the CAM,
experimental work by Burnet and Fenner with ectromelia virus relating to
pathogenesis, biochemical studies of vaccinia infection of the incubated
chicken egg by Caspersson and Thorsson, stressing virus and cellular
metabolism, electron-microscopic observations of fowlpox, canarypox,
mousepox and vaccinia viruses by Ruska, Ruska and Kausche, Morgan and
Wykoff, Wykoff, Gylord and Melnick, Peters and Nasemann, and, more recently,
Herzberg and Kleinschmidt's inquiry into viral morphology and viral propa-
gation.

Material and methods

1. Virus strains

The most important representatives of animal pox viruses, including
vaccinia, cowpox, mousepox, chickenpox and pigeonpox viruses, were
studied comparatively. For technical reasons, sheeppox viruses could
not be included for the time being.

Vaccinia viruses were represented by dermal vaccinia strains CVM,
CVAe and CVA, neurolapine strain NCVL Levaditi and the testicular lapine
strain CVM Nelis (Brussels). The dermal strains are vaccines from
various domestic and foreign lymph production plants and are available
commercially as "smallpox vaccines." The vaccinal strains Levaditi and
Nelis are original virus-fixing strains from the Institute A. Fournier,
Paris. The neurolapine has been received as cerebral material, the
testicular lapine as testicular material. Cowpox viruses included strain
KPH$_1$ and KPH$_2$. Both were isolated between 1944 and 1951 during the last
cowpox epidemics in Holland and England, and adapted to the chicken egg.
Strain KPH$_1$ was supplied by the Dutch Institute for Preventive Medicine
as the 12th egg passage, strain KPH$_2$ as the 8th egg passage.

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Ectromelia strain EPH was used as mousepox virus. This strain was also received from the Dutch institute and was in its first egg passage.

The chickenpox virus under investigation had been isolated in 1954 from an epizootic near Freiburg. The starting material, heads of hens with pox diphtheria, came from the Institute for Veterinary Hygiene at Freiburg. The isolated virus passed through eggs twice before the present work began. It is designated chickenpox strain HPF.

Strain TPB served as pigeonpox virus. It constituted a second egg passage and came from the Behring Works.

The starting material was stored at minus 20°C. During the time covered by these tests, the strains were subjected to up to 20 egg passages.

2. Egg material and incubation

Eggs were procured from white Leghorn maintained by a poultry farm free of epizootics. Care was taken not to include hens vaccinated against fowlpox. Incubators worked on the surface and cabinet (or motor) principles. The working temperature during pre-incubation was 38.5°C (measured on the egg's upper edge) for the surface incubator and 37.8°C (measured in the center of the egg) for the cabinet incubator. Temperatures during the infective period varied in accordance with virus and the purpose of inoculation (cf. Table 1).

Tolerance of adjustment in both systems amounted to 4/10 to 6/10 degrees C and relative humidity was maintained at 50-60%.

3. Virus culture methods

a) General. All strains of virus were injected into the CAM (ectoderm) and, for comparison, into the allantoic cavity (entoderm).

The 10th day of incubation was selected for infection in both methods of inoculation.

The dose of inoculum amounted to 0.1 cc per egg in each case. In comparative studies the virus suspension was adjusted to the same infective titer. We inoculated parallel, ascending dilution series in all tests in order to study the infective course following application of large, medium and small doses.

On the day of inoculation, eggs were culled out, margins of air sacs as well as special markings were drawn, after which they were returned to the incubator. They were inscribed shortly before inoculation, the shell was disinfected with iodo-alcohol, and drilling was carried out. A small dental drill with a corundum disc was utilized.

Inoculation was performed in an "inoculation box." The air contained therein was sterilized before and during injection with a built-in UV tube. Possible contamination of the starting material necessitated addition of penicillin-supronal. It was found to be advantageous that the antibiotics did not lose their efficacy from the time of penetration into the embryonal appendages until hatching, and that they become toxic only in relatively large doses. Streptomycin became toxic for 8-day incubated chicken eggs in concentrations above 30 mg. This made it possible to maintain a bacteriostatic level which prevented multiplication of accidentally introduced bacteria without harming embryonal development.

b) <u>Inoculation of the CAM</u>. Injection followed the method of egg fenestration described previously (1954).

c) <u>Inoculation of the allantoic cavity</u>. This was carried out according to Mayr's method, technique 1.

In inoculations of the allantoic cavity, the injecting cannula must pass through the CAM. The infected needle tip may deposit germs on the membrane in its forward and backward travel, producing simultaneous infection of the membrane.

In order to reduce the percentage of simultaneous membrane inoculations, the cannula was changed after every egg, so that injections were carried out invariably with a dry cannula. Prior to withdrawal of the cannula from the cavity, we rinsed with a small amount of sterile physiological saline. In spite of these precautions, simultaneous CAM infections could not always be avoided.

A simultaneous membrane inoculation is easily recognized in the case of pox viruses. A primary focus develops at the site of injection.

In comparative tests and evaluations we withdrew all those eggs from the test that showed a primary focus upon injection of the allantoic cavity, since there was a possibility that the virus had reached the circulation not via the entoderm, but through the ectoderm or mesoderm. So far our tests have shown no indications that infection of the CAM with pox viruses is possible without formation of foci.

Frequently we saw a small, non-specific nodule of proliferation at the site of CAM injection, which had developed over the stab injury. These nodules of proliferation are readily differentiated from primary pox foci, both macroscopically and microscopically.

4. Procedure after inoculation, and harvesting
of virus material.
Cf. Herrlich and Mayr, Arch. Hyg. 138:479 (1954).

5. Treatment of individual embryonal organs for
virus titrations and passages.

Raw materials were ground in the mortar 1:5 with sterile physio-
logical saline and sterile sand, then centrifuged for 15 minutes at
4000 RPM. Allantoic and amniotic fluids were also spun at 4000 RPM after
repeated climatic flection. The supernatant served as standard solution.
Egg passages were conducted exclusively with standard solutions obtained
by CAM homogenization. Only suspensions free of bacteria were injected.

6. Virus titrations.

Virus titrations were applied to 0.1 cc of standard solution.
Testing of all strains was accomplished by inoculation of dilution series
on the CAM of hen's eggs incubated for 10 days. Evaluation was based on
the "pox count method" developed by Burnet and Faris. Vaccinia and cowpox
suspensions were tested comparatively in cutaneous infection of the rabbit
after Herzberg and in intracutaneous injection according to Groth.
Ectromelia dilutions were tested by cutaneous inoculation of the tail of
white mice, and chickenpox as well as pigeonpox suspensions by cutaneous
application to the hen's comb and throat flap.

7. Adaptation of the starting material to
the incubated chicken egg.

In the case of vaccinias, the cerebral and testicular material
(or the pustular material from combs infected with chickenpox) was
ground in the mortar 1:5 with sterile, ice-cooled physiological saline
and sterile sand. It was then centrifuged for $\frac{1}{2}$ hour at 4000 RPM.
Dermovaccines were spun in the same manner. The supernatant was employed
as trans-inoculum. Contamination with accompanying germs necessitated
addition of penicillin-streptomycin.

The inoculum was applied to the CAM of eggs pre-incubated for 10 days
in dilution series from 10^{-1} to 10^{-6}.

Depending on the infective titer, embryos infected with low dilutions
of vaccines died within the first few days. Dilutions with terminal
titers did not kill embryos within this term. Generalization developed
instead. Generalized foci on the chorioallantois furnished material for
further experiments.

Chickenpox material produced typical membrane lesions only after 3 passages. Following the 4th membrane passage they also evoked generalization at high dilutions. Generalized foci of the 4th passage were used as starting material.

Strains already adapted to the egg were passed through eggs twice before the present comparative studies were started. Primary lesions of the second passage served as starting material. Specificity of lesions was demonstrated in parallel tests.

8. Bacteriological tests.

Only standard solutions of virus were used for bacteriological tests. Two batches of 0.05 cc each were applied simultaneously to blood agar, Gassner plate, dextrose nutrient broth and liver-liver broth.

Results

1. Common characteristics.

Inoculation of the CAM of 10-day incubated hen's eggs with all available strains of vaccinia, cowpox, ectromelia, chickenpox and pigeonpox viruses produced the same picture in the type and course of pathogenetic chain from infection to the first tissual reaction and onward to the complete symptomatology. Together with the primary settlement of virus at the site of injection, the infection developed in the liver, spleen and bone marrow, producing generalization in embryonal and extra-embryonal organs. Primary invasion and parallel infection of RES organs is designated "primary virus phase" by us, the ensuing generalization as "secondary virus phase." The course of individual phases was dependent both upon the virus and its virulence as well as on its host and the host's defensive power. The secondary phase does not always develop, and when it does, it does not always have the same intensity.

Fenner has shown with ectromelia-infected mice that an intermediate phase exists prior to generalization: During incubation, the virus reaches the blood from the portal of entry. It is enclosed intracellularly in the RES, especially in the liver. The virus multiplies vigorously and generalizes after having overcome tissual barriers. Generalization is dependent upon the process of propagation on one hand, and upon cell destruction on the other.

Herrlich and Mayr demonstrated the same biphase course experimentally in the egg in connection with various vaccinal strains and cowpox virus. The present study confirms this pathogenesis for ectromelia and chickenpox virus and supplements the above-mentioned findings in the case of vaccinia and cowpox virus. The agreement of Fenner's mouse test with our results with eggs suggests that the course of other animal pox viruses in their natural hosts follows laws established with the aid of eggs.

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This result necessitates revision of current concepts of pathogeny and epidemiology of animal pox. At the same time we get remarkable parallels to human variola infections, as already pointed out by Herrlich and Mayr. In man, invasion of the respiratory tract by pathogens should be followed by initial viremia with settlement and propagation in the RES. Only then does secondary viremia set in, accompanied by clinical symptoms with viral manifestations on the skin and mucous membrane.

All tested animal pox viruses adhered to the entoderm (inoculation of allantoic cavity). However, the ability to persist (capability of active cell membrane passage) was quite variable. In the case of vaccinia virus it resembled that of the ectoderm, in ectromelia virus and fowlpox it was somewhat reduced; it was quite poor in the case of cowpox virus. When infection set in, the disease progressed in phases identical to those following membrane inoculation. In the case of cowpox virus, infections of liver, spleen and bone marrow, and concomitant generalization were markedly less severe.

The histopathology of animal pox infections of the egg was characterized by primary lesions in affected epithelium, by inflammatory processes arising subsequently in the proper mesenchyma, by deleterious affection of vessels and by focal proliferation, degeneration and necrosis in the affected internal organs. Primary lesions were related intimately with the activity of virus and its intracellular reproduction. The cell died whenever viral propagation in the cell was very rapid (vaccinia and cowpox). Primary changes in the case of all animal pox viruses consisted initially of local hyperplasia, followed by ballooning, vacuolate or reticulate degeneration of the proliferate cell, during which cytoplasmic inclusion bodies are formed.

The sequence of events varied among viruses. Tissue destruction was contrasted with behaviour indifferent to tissues. Vaccinia and cowpox virus produced necrosis in the altered epithelial sections which began in the center and spread with variable rapidity, at times into the mesoderm. In the case of fowlpox viruses, cellular proliferation continued without resultant necrosis. In that event the virus-infected cell, although subject to pathological changes, retained its connection with the tissue. The affected organs were frequently enlarged to twice their size and altered by focal proliferation, degeneration and necrosis with variable involvement of the vascular system. The liver revealed diffuse necrosis or more circumscribed necrotic foci, cellular infiltration and changes of variable extent in the walls of vessels.

All animal pox viruses seem to contain a component injurious to vessels. It was demonstrated in a number of vaccines, distinctly in cowpox virus, in ectromelia virus (especially in internal organs), but could not be shown clearly in chickenpox virus, although it reappeared in pigeonpox virus in particularly susceptible hosts.

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The titer values of the allantoic and amniotic fluid were unusually low in all phases of the infective course of tested animal pox viruses. They did not rise even upon inoculation of the allantoic fluid, or after generalization. This behavior, characteristic of animal pox viruses, may be connected with the development of inclusion bodies and the focal defensive reaction of the host's tissue. We know today that these inclusion bodies contain massive virus elementary bodies. It is possible that the virus is constrained by inclusion bodies and the host's defensive mechanism, and cannot be released from its site of propagation on the scale we know of influenza group viruses. Thus the amniotic and allantoic fluid is unsuitable for a quantitative harvest of pox viruses.

The common biological properties of examined animal pox viruses, as observed in the egg, confirm the close relationship of all pox viruses. They solidify their position in the system and, consequently, their classification in the virus group with the group designation pox virus.

2. Constant differences which may be reproduced in all passages.

In spite of common factors, individual animal pox viruses are not identical, but give the impression of independent and stable viruses that are readily differentiated in all passages through incubated hen's eggs by their unaltered biological properties. In this connection inoculation of the CAM generally is better suited for differential diagnosis than infection of the allantoic cavity. The latter does, however, offer a good medium for the differentiation of vaccinia and cowpox viruses. Dissimilarities are revealed consistently and positively on the CAM. They were parallel to other differences observed, e.g., to mortality curves, settlement of virus in the internal organs and to titers obtained from tissue during the various infective phases, although the membrane offered a clearer picture as a matter of routine. Differences established on the membrane referred to:

a) incubation time,

b) the nature of ectodermal lesions of primary and secondary foci,

c) position and development of inclusion bodies,

d) type and extent of inflammatory processes in the mesoderm sequential to epithelial processes,

e) the time at which secondary settlement became visible on the membrane, and

f) the percentage of generalization and its quantity.

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In addition, we found constant and evaluable differences in the intensity of reproduction among individual viruses. Dissimilarities suited for differential diagnosis are listed in Table 1. They permit positive and uncomplicated differentiation of vaccinia, cowpox, ectromelia, fowlpox and pigeonpox viruses.

Downie has demonstrated the differences between vaccinia and cowpox viruses. Herrlich and Mayr obtained the same results in their latest study. Moreover, they confirmed the independence of these two viruses in rabbit tests. In the present study, cowpox virus is again delineated clearly from vaccines used for human immunization (cf. Table 1a). Vaccinia virus was characterized by rapidly ensuing cellular disintegration with much cellular detritus and by relatively numerous, but small, granular inclusion bodies. Cowpox, on the other hand, produces violent and persistent epithelial proliferations, delayed central lysis, and large, compact, homogeneous and less numerous inclusions. The inflammatory reactions in the wake of epithelial processes were hemorrhagic in the case of cowpox virus, in contrast to vaccinia virus.

In addition to differentiation on the CAM, dissimilar behavior upon inoculation of the allantoic cavity is remarkable. Vaccinia virus adhers to the entoderm as well as to the ectoderm; cowpox virus, on the other hand, asserts itself quite poorly. Nevertheless, these two viruses were far closer to each other in their biological expressions than to ectromelia and fowlpox viruses. They shared the relatively broad, areal nature of focal membrane lesions and consistent, centrally commencing cellular disintegration which set in at variable intervals following proliferation of epithelial cells. Both viruses produced inclusion bodies in all three germinal layers. Both viruses multiplied very rapidly and intensely; titers of 10^{-8} to 10^{-9} in affected tissue sections were by no means rare. Generalization was the rule. Its course was violent, but differed quantitatively among strains. Not one egg survived the infection. Embryos died during the primary virus phase after inoculation of concentrated virus suspensions, whereas high dilutions produced death during the secondary phase.

Among vaccines, the first impression was a very consistent chorioallantoic picture. The hemorrhagic component frequently described in connection with neural and testicular vaccines, especially in rabbits, could not be duplicated in egg passages. The neural and testicular vaccines therefore do not produce reactions fundamentally different from dermal vaccines. However, other differences were considerable. One strain generalized constantly through all passages after 72 hours, another became visible on the CAM only after 96 hours. The quantity of generalization fluctuated from strain to strain. Inflammatory lesions on the membrane also diverged. They may have been changed occasionally by the host organism. In addition, attention is directed to the strains' variable effect on vessels. Most remarkable was the variable behavior of individual strains with respect to the appearance of "cutaneous pox" in the

course of generalization. One strain formed them consistently and in great number, another less so, and some strains not at all. The essential properties of vaccines in the egg, i.e., intensity of propagation, vascular involvement and cutaneous affinity, were not always parallel. Observed properties were retained through all egg passages. We may assume, on the basis of their nature, that stable virus variants are involved. It is unimportant in practice whether the change is called a variation, modification or mutation. It is significant, however, that strains currently used for human immunization have shown constant differences with respect to propagation tendencies, vascular involvement and cutaneous affinity.

Ectromelia viruses were readily differentiated macroscopically from vaccinia, cowpox, chickenpox and pigeonpox viruses on the chorioallantois (cf. Table 1b).

When culturing ectromelia viruses for the purpose of differential diagnosis vis-a-vis other animal pox viruses, the incubation temperature is particularly important. According to Burnet, the optimal incubation temperature for ectromelia viruses is between 35 and 37°C (surface incubator), i.e., about 1.5 to 3.5°C lower than the physiological temperature of eggs. Ectromelia viruses multiply best under these conditions. Low temperatures promote the activity of the virus, which is less "active" in the egg, and, according to Burnet, lead to complete necrosis of the affected ectoderm with particularly strong development of inclusion bodies in the mesoderm, since the natural defenses of the embryo are greatly inhibited by the unphysiological temperature. Incubation at 36°C is recommended for ectromelia virus whenever an optimal quantitative yield is desired. It is less favorable for differential diagnosis. Incubation at temperatures customary for vaccinias has been found most suitable.

As in the case of vaccinia and cowpox viruses, strains of ectromelia virus are differentiated best by the time at which visible secondary settlement takes place on the CAM. Incubation at 36°C is recommended for strain identification under optimal conditions of propagation. Generalization appears earlier on the membrane at 36°C.

Differences inherent in chickenpox and pigeonpox viruses were greatest in relation to vaccinia and cowpox as well as to ectromelia viruses. Fowlpox viruses were fundamentally different from other animal pox viruses in their chorioallantoic picture, a doubled incubation time, extraordinarily low virus content in morbid tissue, in the high survival rates of infected embryos, very fatty inclusion bodies, benign involvement of tissue and not least in the slow, non-acute progress of the disease (cf. Table 1c).

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Aside from the dissimilar macroscopic and histological appearance of fowlpox foci, the slow course of this disease in the egg is remarkable. Apparently it is connected directly with the intensity of propagation of which these viruses are capable. Comparative titrations of embryonal and extraembryonal organs yielded values in the case of fowlpox infections which were much lower than those associated with mammalian pox. Thus CAM titers never rose above 10^{-4}, even after generalization, whereas vaccinia and cowpox viruses yielded between 10^{-8} and 10^{-9}; ectromelia virus produced between 10^{-5} and 10^{-6}. Titer values in other organs were similar. Comparative quantitative titrations of confluent primary lesions from initial appearance of visible changes to their apex show the same variable values. In fowlpox, the titer maxima were invariably about 2 to 3 decimal powers lower. When the amount of virus found in organs in the various developmental stages is related to the intensity of propagation of the virus, we obtain the following scale of evaluation: Vaccinia and cowpox viruses multiply most rapidly in the incubated hen's egg, the chickenpox viruses are slowest, the ectromelia viruses occupy an intermediate position. These circumstances impede quantitative culture of fowlpox viruses in the incubated chicken egg. Individual harvests must be concentrated enormously before quantities comparable to mammalian viruses can be obtained. This is particularly bothersome in the production of antigen for serological studies. Incubation at $36^{\circ}C$ promotes viral activity; generalization sets in earlier and is increased quantitatively. Virus harvests also increase slightly, but do not attain values comparable to mammalian pox even under optimal conditions.

Experimental pigeonpox infections resembled essentially those of chickenpox. The times at which visible secondary foci appeared on the CAM were different, as were the percentage and intensity of generalization, the course of mortality curves and the mesodermal reaction. Pigeonpox virus produced sporadic and unreproducible vascular damage in susceptible hosts. This permits demonstration of quantitative and qualitative differences between chickenpox and pigeonpox viruses in the course of experimental infections of the incubated chicken egg.

According to Bierbaum, the virus of fowlpox does not constitute a closed entity, but represents three types differentiated by clinical, immunological and histologic-anatomical properties: chickenpox virus (HPV), pigeonpox virus (TPV) and sparrow or canarypox (KPV). The nomenclature at the same time identifies the avian species for which the virus is specific. Bierbaum is not certain whether additional viruses occur among birds. According to Bierbaum and Eberbeck, the histological appearance of fowlpox foci not only permits their differentiation from other animal pox, but also separation of individual fowlpox viruses. As a rule, HPV, TPV and KPV assert themselves only on the homologous avian species and evoke specific lesions in the epithelial cells of the epidermis.

The effect of heterologous virus, on the other hand, is said to be directed more against the mesenchymal tissue of the chorion. Bierbaum and Weitzenberg found, however, that TPV may produce specific lesions on the epithelial cells of the chicken, with formation of inclusion bodies. The authors concluded that fluent transitions exist among all three types of virus, a fact which is not surprising when their close relationship is considered. The present study with the aid of 10-day incubated chicken eggs generally confirms these findings. The common reactions observed in the egg also support a close relationship of the two viruses. It is likely that canarypox virus (not tested by us) also fits this uniform morbid picture. At the same time, new possibilities of differentiation among fowlpox viruses and between the latter and the remaining animal pox viruses were indicated.

3. Methodology.

A critical examination of method must first establish the influence of the host organism on the pathogen's functional aspect. Compared to the customary laboratory animals, the egg seemed to be a uniform host with respect to constitution, disposition and resistance.

For purposes of comparative differentiation of viruses in eggs incubated for 10 days, individual animal pox viruses should not be incubated at their optimal incubation temperature, but should be grown at identical temperatures, i.e., at $37.8^{\circ}C$ in the cabinet incubator and at $38.5^{\circ}C$ in the surface incubator. Differences crystallize better under these conditions. The appearance of the CAM after membrane inoculation generally suffices for differentiation.

Identification of strains is accomplished best by way of chronological differences in the ability to generalize in the egg, and by the quantity of resultant generalization. It is easily read on the CAM after inoculation of a correspondingly small amount. The host had no influence on this criterion. It is recommended, however, to culture the virus strains to be differentiated at their optimal temperature. In the case of ectromelia and fowlpox, that would be $36^{\circ}C$. In evaluating the chronological difference in generalization on the CAM (ZG), greater accuracy is obtained when the time at which at least 50% of eggs are visibly generalized (ZG/50), instead of all inoculated eggs (ZG/100), is established. Computation of ZG/50 should be done according to the statistical principles proposed by Reed and Muench (Behrens) for LD 50 and ID 50.

Identification of strains is important in practice principally in connection with vaccinia virus and its evaluation as an immunogen. Herzberg, Herrlich and Mayr have called attention to possible correlations between vaccinia strains and the course of human vaccination. Their experimental work was based on the concept that the vaccine itself is one of the factors responsible for abnormal vaccinal processes. In the past it has been denied that the lymph participates in the genesis

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of vaccinal complications. Paschen was one of the few immunizers who
included vaccinia virus in the causative complex of vaccinal damage.
It would be interesting to know the extent to which results obtained by
Herzberg, Herrlich and Mayr in animals and chick embryos may be compared
to the human condition. We know from the histological studies of Kruecke
and Schleussing that the vaccinal process at times involves the blood
vessels and that various inflammatory processes are found, depending on
the lymph. It is not improper to assume that such findings, established
in animals, may occur in the vascular system of man. The diagnosis of
strains, as developed in this study, is designed to help clarify the
discussed problems.

The importance of strain diagnosis of other viruses, perhaps in
vaccination against fowlpox, cannot be estimated. Considering the great
plasticity of animal pox viruses, identification may aid in the establish-
ment of biological changes.

Occasionally the chorioallantoic picture characteristic of the
appropriate virus becomes typical only after several egg passages.
Moreover, it may have been altered by non-specific accompanying substances
of the inoculum, especially during initial passage. Involvement of the
host has been mentioned already. Special attention must be directed to
the effect of variable concentrations. High concentrations generally lead
to confluent lesions, low concentrations allow development of isolated
foci. For this reason definitive diagnosis requires several egg passages
with different dilutions. Inoculation of the CAM usually was better
suited for differential diagnosis than injection of the allantoic cavity.
Differentiation between vaccinia and cowpox, and determination of possible
vasopathogenic components among the various strains require the addition
of allantoic cavity infection.

The pathway of infection after inoculation of the allantoic cavity
generally proceeds via adsorption of virus on the entoderm of membranes
that form the allantois. As already shown, a second route of infection
must be considered. The injecting cannula perforates the CAM in every
case before reaching the allantoic cavity. The infected needle tip may
deposit organisms cn the CAM during withdrawal from the cavity, producing
simultaneous infection of the membrane. The percentage of simultaneous
membrane infections may be reduced, but never eliminated, by working with
dry cannulas after every egg, by short aspiration of the syringe after
injection while still in the allantois, by rinsing with physiological
saline, etc. Pox viruses always produced a small, confluent primary
lesion at the site of injection when infection was simultaneous.
Frequently a non-specific nodule of proliferation was observed at the
site of injection.

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In the case of vaccinia, cowpox and ectromelia viruses, death of eggs during the first 24 hours p.i. must be considered non-specific. The same is true of fowlpox viruses during the first 48 hours post infectionem.

One other result of our study should be pointed out. The survival time of the embryo usually was inversely proportional to the amount of injected virus. Whenever embryonic death during the primary virus phase was prevented by very low doses (especially valid for socalled "active" viruses), generalization in the embryo and the embryonal appendages invariably set in. This permits harvesting of considerably higher amounts of virus from the CAM and other membranes, e.g., the inner skin of the allantois, the allantoic septa, from the amniotic layer, and from the whole embryo. Thus the total virus harvest is increased manifold, a circumstance of particular importance for vaccine and antigen production.

Plan for the differentiation of animal pox viruses in the incubated hen's egg. (Tables 1a, 1b and 1c)

Table 1 (1a, 1b, 1c) lists differences demonstrable in the incubated chicken egg that are suitable for the differentiation of animal pox viruses (virus diagnosis). It is important that all pox viruses be grown at identical temperatures -- at 37.8°C in the cabinet incubator, at 38.5°C in the surface incubator. The biological data of Table 1 refer to this incubation temperature in the case of CAM and allantoic cavity injection.

In addition to virus diagnosis, Table 1 makes brief reference to the possibility of strain diagnosis. When differentiating strains, it is recommended to grow viruses at their optimal temperatures, which are lower for ectromelia and fowlpox when compared to vaccinia and cowpox.

Legend

ZG 50 = time of visible generalization on the CAM at which at least 50% of all inoculated eggs are generalized.

Quantity of generalization.

/ = not more than 8 secondary foci.
// = between 8 and 30 secondary foci.
/// = between 30 and 100 secondary foci.
//// = the whole membrane is covered with closely arranged secondary foci.

Tendency of propagation.

$\not{/}$ = titers of affected membrane regions at the apex of primary virus phase not over 10^{-3}.

$\not{/\!/}$ = not over 10^{-4}.

$\not{/\!/\!/}$ = between 10^{-5} and 10^{-6}.

$\not{/\!/\!/\!/}$ = between 10^{-6} and 10^{-9}.

Illustrations

Fig. 1. Vaccinal lesions on the 4th day p.i. with dermal strain CVAe. Inoculum 20 MID. Secondary foci are still small; central necrosis has already developed.

Fig. 2. Ectromelia lesions on the CAM on the 3rd day p.i. with low concentration. Foci have developed individually and are distributed diffusely over the membrane. They consist of small, compact, slightly raised nodules surrounded concentrically by a narrow zone of turbidness. The membrane is somewhat cloudy between foci. No central necrosis.

Fig. 3. Ectromelia lesions on the CAM on the 4th day p.i. with high concentration. Individual foci have fused into a wide, communicating, sharply delineated primary cover. The cover is slightly loosened at the edge, revealing small, compact, densely packed foci of ectromelia.

Fig. 4. Appearance of chorioallantois on the 5th day p.i. with mouse-pox virus; small, confluent primary lesions; $\not{/\!/\!/}$ generalization along the vessels.

Fig. 5. Appearance of chorioallantois on the 3rd day to 4th day p.i.; high concentration. Confluent lesions.

Fig. 6. Appearance of chorioallantois on the 5th day p.i. with chickenpox virus, strain HPF. Primary foci are typically constructed: concentric zone of turbidness around a compact, raised, wide, round, white focus. Commencing generalization.

Fig. 7. Appearance of chorioallantois on the 7th day p.i. with chickenpox virus; high concentration, $\not{/}$ to $\not{/\!/}$ generalization.

Fig. 8. Appearance of chorioallantois on the 7th day p.i. with chickenpox virus; low concentration, $\not{/\!/}$ to $\not{/\!/\!/}$ generalization.